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Birth of normal young after electrofusion of mouse oocytes with round spermatids

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ABSTRACT **Normally, round spermatids, the youngest male germ cells with a set of haploid chromosomes, cannot fertilize mature oocytes. However, when mouse spermatids were fused with oocytes, some of the resulting zygotes developed into normal fertile mice of either sex. This demonstrates that the nuclei of spermatids can provide the paternally imprinted chromosomes needed for full embryonic development and also that the complex postmeiotic modifications involved in sperm formation in the testis and their maturation in the epididymis merely serve to facilitate natural delivery of the paternal genome. This finding may find an application in the treatment of male infertility due to defective spermiogenesis/sperm maturation.**

Male and female gametes both contribute to the genome of the zygote, but their morphology and behavior prior to fertilization are quite different. Generally, mammalian oocytes are released from the ovary during metaphase of the second meiotic division, which is stimulated to completion by the fertilizing spermatozoon (1). Male germ cells, on the other hand, complete their meiosis long before fertilization, and the youngest haploid stage, the round spermatid, then undergoes a series of profound structural and biochemical modifications in the testis and epididymis to become a functional spermatozoon. However, since the spermatid nucleus contains a haploid set of chromosomes that are almost certainly imprinted (2–5), one might expect zygotes of full developmental potential when such nuclei are introduced directly into the cytoplasm of mature oocytes. We have previously reported that the nuclei of round spermatids from hamsters and mice are able to duplicate DNA and participate in syngamy when incorporated into oocytes by either microsurgery (6) or electrofusion (7). Here, we report that some of the mouse zygotes thus produced develop into normal males and females.

MATERIALS AND METHODS

Preparation of Spermatids and Oocytes. Round spermatids were isolated from the testes of B6D2F$_1$ or C57BL/6 mice (both with black eyes and black coats) as described (6, 7) except that all handling was performed at about 10°C to reduce the incidence of cell lysis. Mature oocytes were obtained from the oviducts of PMSG-primed superovulated females of B6D2F$_1$ mice 17–18 hr after human chorionic gonadotropin injection. The oocytes were freed from cumulus cells by a 2-min treatment with 300 units of bovine testicular hyaluronidase per ml in Whitten's medium (8) buffered with 12.6 mM $NaHCO_3$ and 10 mM Hepes (Hepes/Whitten medium). Cumulus-free oocytes were transferred to Dulbecco's phosphate-buffered saline (PBS) containing 0.5 mg of 360-kDa polyvinylpyrrolidone (PVP) per ml.

Oocyte Activation and Electrofusion with Spermatids. Oocyte activation prior to application of fusion pulse is known to facilitate oocyte–sperm fusion (7). The oocytes in Dulbecco's PBS were electroactivated by consecutive exposures to ac (2 MHz, 20–50 V/cm, 10 sec) and dc (1500 V/cm, 80 μsec) pulses using an SSH-1 somatic hybridizer (Shimadzu, Japan). The oocytes were transferred back to the Hepes/Whitten medium and a single spermatid was injected into the perivitelline space of each oocyte as described (6, 7) (Fig. 1*A*). The oocyte–spermatid pairs were placed in fusion medium (300 mM mannitol/50 μM calcium lactate/100 μM $MgSO_4$/0.5 mg of PVP per ml) and exposed to a fusion dc pulse (3750–4000 V/cm, 10 μsec) preceded and followed by application of ac pulses (2 MHz, 100 V/cm, 15–30 sec each). The time interval between application of the oocyte activation pulse and the oocyte–spermatid fusion pulse was 15–40 min. Electrofused oocytes were incubated in the regular Whitten's medium (8) under 5% CO_2 in air for up to 20 hr. Some oocytes were fixed 5 hr after onset of incubation and stained to examine the state of spermatid and oocyte pronuclei (9).

Embryo Transfer to Foster Mothers. Foster albino females (ICR strain) were mated with a vasectomized male of the same strain that had been proven infertile by previous mating. About 12 hr after mating, 11–30 two-cell embryos were transferred, with minimum volume of associated medium, into the ampullary region of each oviduct using a micropipette inserted into the oviduct through the infundibulum. Foster females were kept individually thereafter.

RESULTS

When 50 randomly selected ova were examined 5 hr after application of the fusion pulse, all of them had extruded the second polar body. Fifteen (30%) of the ova contained a large female pronucleus and a small male pronucleus, the latter derived from the spermatid nucleus (Fig. 1*B*). In the remaining oocytes, spermatids, either intact or degenerating, were in the perivitelline space, and each oocyte contained only a solitary female pronucleus. A total of 475 ova was incubated for 20 hr, at which time 451 (95%) had cleaved to the two-cell stage. This was comparable to the cleavage rate observed in control ova electrostimulated without spermatids.

By ordinary light microscopy it was impossible to determine which two-cell embryos were derived from fused oocyte–spermatid pairs and which were from unfused pairs because of disintegration (disappearance) of unfused spermatids. Therefore, we transferred all of the 346 normal-looking two-cell embryos into the oviducts of eight pseudopregnant ICR albino mice. Three of these mice produced six young, of which four (two female and two males, with black eyes and black coat) survived (Fig. 2*A*). Since the albino foster mothers had never been exposed to pigmented males or their spermatozoa, and since full parthenogenetic

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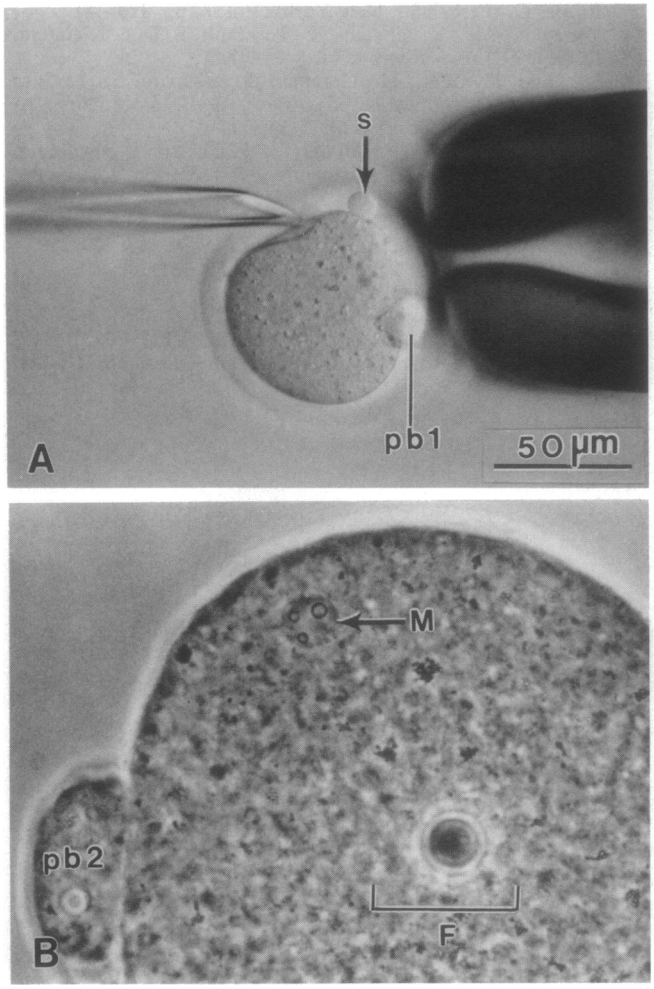

FIG. 1. (*A*) Spermatid (s, arrow) injected into the perivitelline space of a mouse oocyte. (*B*) Oocyte 5 hr after electrofusion with a spermatid; the zona pellucida had been dissolved during fixation due to the acidity of the fixative. pb1, First polar body pb2, second polar body; M, small male pronucleus derived from spermatid nucleus; F, female pronucleus derived from oocyte's chromosomes, out of focus in this optical section except for a large nucleolus.

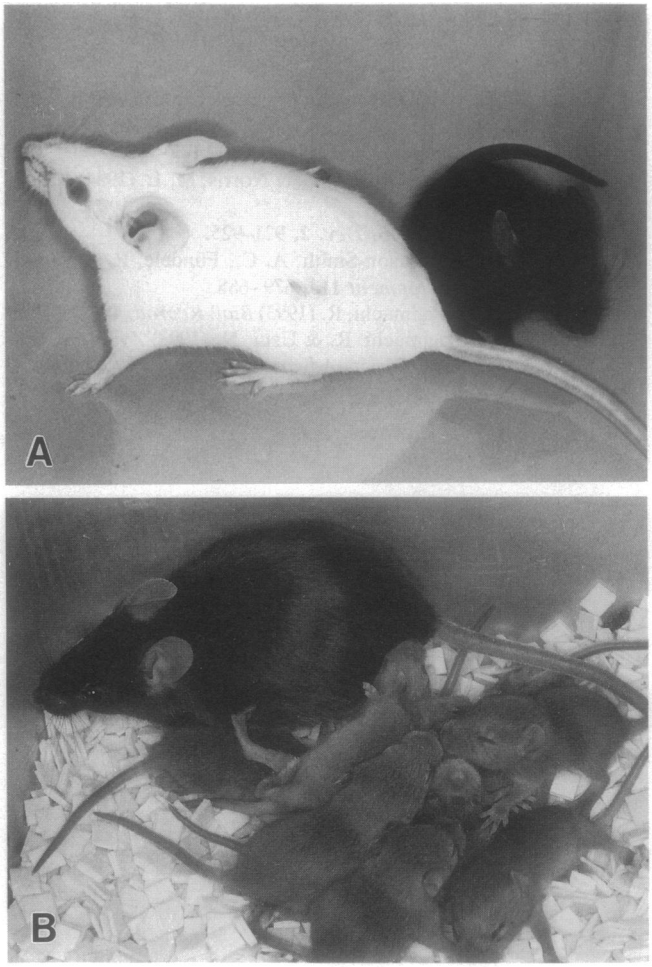

FIG. 2. (*A*) Albino mouse (ICR) with her foster black pup developed from an oocyte fertilized by a spermatid (both from B6D2F$_1$ mice). (*B*) The above black mouse mated with an albino ICR male gave birth to offspring of a normal litter size.

development together with a black mutation at the *c* locus are both extremely unlikely, the black offspring must have been derived from the oocytes fused with round spermatids. The four offspring grew normally. When paired with albino ICR mates of the opposite sex they produced pigmented (agouti) offspring of normal litter size (Fig. 2*B*). This shows that the original progeny were homozygous for genes for pigmentation and thus even less likely to have developed from a mutation.

DISCUSSION

We have now demonstrated that when a mature oocyte is artificially fertilized by a round spermatid the resultant zygote is capable of normal development. From this we may conclude that the nuclei of round spermatids, like those of mature spermatozoa, have reproductive potential and, further, that all or most of the postmeiotic events (spermiogenesis, epididymal maturation, capacitation, and acrosome reaction) (10–12) may have evolved just to ensure the delivery of competent male haploid nuclei into oocytes. This finding provides experimental proof of what previously has been purely theoretical conjecture.

Although the success rate reported here is very low, experiments nevertheless show that there is no genetic barrier to fertilization by round spermatids. It is probable that a much better rate of success can be obtained if, in the future, many of the variables involved are controlled. These variables include the animal genotype, media, handling temperature, magnitude and timing of activation and fusion pulses, and time taken for manipulation of the oocyte–spermatid pairs and embryos. The great differences we have observed between mice and hamsters (6, 7), particularly in regard to embryo culture and survival, indicate that many other subtle factors contribute to the final outcome.

The preliminary results reported here may offer exciting prospects for the treatment of male infertility due to defective spermiogenesis. It seems likely that if spermatids can be obtained in an acceptable way from azoospermic patients, these cells can be used to construct zygotes with full developmental potential. Although one may wonder whether some cases of azoospermia may have a genetic basis, the majority of azoospermic men are at least karyotypically normal (13–16). Thus, except for cases where genetic defects are known to be involved, consideration should be given to the approach employing spermatid–oocyte fusion (or microinjection of spermatid nucleus into the oocyte) as a means of overcoming male infertility due to defective spermiogenesis.

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